A TOBACCO-SPECIFIC LUNG CARCINOGEN IN THE URINE OF MEN EXPOSED TO CIGARETTE SMOKE

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Abstract *Background.* Environmental tobacco smoke has been classified by the Environmental Protection Agency as a carcinogen causally associated with lung cancer in adults, but there have been no reports of lung carcinogens or their metabolites in the body fluids or tissues of nonsmokers exposed to environmental tobacco smoke.

Methods. Five male nonsmokers were exposed to sidestream cigarette smoke generated by machine smoking of reference cigarettes for 180 minutes on each of two days, six months apart. Sidestream smoke is the smoke that originates from the smoldering end of a cigarette between puffs. Twenty-four-hour urine samples were collected before and after exposure. The urine samples were analyzed for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronide, which are metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a powerful lung carcinogen in rodents. NNAL is also a lung carcinogen in rodents.

Results. The urinary excretion of the metabolites increased after exposure to sidestream smoke in all the men. The mean (±SD) amount of NNAL and NNAL glucuronide was significantly higher after exposure than at base line (33.9±20.0 vs. 8.4±11.2 ng per 24 hours [127±74 vs. 31±41 pmol per day], $P<0.001$) and was correlated with urinary cotinine excretion ($r = 0.89$, $P<0.001$). The nicotine concentrations in the air to which the men were exposed were comparable to those in a heavily smoke-polluted bar.

Conclusions. Nonsmokers exposed to sidestream cigarette smoke take up and metabolize a lung carcinogen, which provides experimental support for the proposal that environmental tobacco smoke can cause lung cancer. (N Engl J Med 1993;329:1543-6.)

ENVIRONMENTAL tobacco smoke has been classified by the Environmental Protection Agency (EPA) as a human carcinogen. A recent report[1] concluded: "Passive smoking is causally associated with lung cancer in adults, and environmental tobacco smoke, by the total weight of evidence, belongs in the category of compounds classified by the EPA as Group A (known human) carcinogens." This evaluation was based in part on dose–response relations between the exposure of spouses to environmental tobacco smoke and the development of lung cancer, especially pulmonary adenocarcinoma.[2] There have been numerous studies of noncarcinogenic tobacco constituents such as nicotine and cotinine in the blood and urine of nonsmokers, and recent studies have established the presence of adducts of hemoglobin and 4-aminobiphenyl, a bladder carcinogen, in nonsmokers exposed to environmental tobacco smoke.[3,4] In this paper, we describe the presence of metabolites of a tobacco-specific lung carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in the urine of nonsmokers exposed to sidestream cigarette smoke. Sidestream cigarette smoke is the smoke that originates from the smoldering end of a cigarette between puffs; it is the principal component of environmental tobacco smoke.

NNK is a powerful pulmonary carcinogen, inducing predominantly adenocarcinomas in the lungs of rats, mice, and hamsters regardless of the route of administration.[5-7] The lowest total doses required to induce lung tumors in rats are similar to the total doses to which smokers are exposed, as calculated from both the amounts of NNK in mainstream cigarette smoke and measurements of its metabolites in the urine of smokers.[8-10] These data, as well as biochemical studies that have demonstrated the presence in smokers' lungs of DNA adducts likely to result from the metabolic activation of NNK, suggest that NNK has an important role in the induction of lung cancer in smokers.[11,12] In primates, NNK is extensively metabolized.[13] Although NNK itself is barely detectable in urine, up to 25 percent of the dose is excreted as glucuronide conjugates of its carbonyl reduction product, 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). NNAL is also a powerful lung carcinogen in rodents.[7,14] Recently, we demonstrated the presence of these metabolites in the urine of smokers.[10] In this study, we measured NNAL, its glucuronide, and cotinine, a metabolite of nicotine, in the urine of nonsmokers exposed to sidestream smoke.

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METHODS

Study Design

We conducted two identical studies, each involving five men who were nonsmokers. The five men in study 1 ranged in age from 29 to 49 years. Four of these men participated in study 2; the fifth subject in study 2 was 57 years old. The protocol was approved by the American Health Foundation's institutional review board for the protection of human subjects, and all the men gave written informed consent. No restrictions were placed on the participants before the studies.

Study 1 was conducted in August 1992, and study 2 in February 1993. Two days before exposure to sidestream smoke, each man collected a 24-hour urine sample for the measurement of NNAL and its glucuronide.

The men were exposed to sidestream smoke in a 16-m^3 room with limited ventilation. There were two smoke-exposure sessions in each study: session 1 lasted from 9:30 a.m. to 11 a.m., and session 2 from 2 p.m. to 3:30 p.m. During each session, the five men sat together quietly in the room while sidestream smoke was generated by a smoking machine. Only sidestream smoke was allowed to enter the atmosphere in the room; mainstream smoke was trapped on a 92-mm Cambridge filter, and the remaining gas phase was pumped out of the room. In session 1, six University of Kentucky 2R1 reference

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cigarettes (nonfilter) were smoked on a Borgwaldt 20-port smoking machine with a rotating head under standard smoking conditions (duration of puff, 2 seconds; frequency, one per minute; and volume, 35 ml), at the rate of two cigarettes simultaneously every 30 minutes. In session 2, 12 Kentucky 2R1 reference cigarettes were smoked under the same conditions, at the rate of 4 every 30 minutes. We used two sessions to avoid prolonged continuous exposure of the men to sidestream cigarette smoke. There was less exposure in session 1 than in session 2 to allow the subjects to become acclimated to the smoke. On the day of smoke exposure, the men collected 24-hour urine specimens beginning at 11 a.m., when session 1 ended. This schedule was chosen so that all urine produced after exposure to NNK in sidestream smoke would be collected, since NNK metabolites are excreted rapidly.

Analyses

The concentrations of nicotine and NNK in the air were determined by standard methods.[15,16] The 24-hour urine samples were analyzed for NNAL and its glucuronide by a modification of our published method, which has interassay coefficients of variation of 9.7 percent for NNAL and 17.1 percent for NNAL glucuronide.[10] Fifty nanograms of 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) was used as an internal standard instead of [5-^{3}H]NNAL.

The pH of the urine was adjusted to 7, and the urine was extracted four times with ethyl acetate. The extracts were combined, the internal standard was added, and the solution was dried and concentrated to produce fraction 1, which contained unconjugated NNAL. The aqueous layer was treated with 500,000 units of β-glucuronidase type IXA (Sigma Chemical, St. Louis). Internal standard was added, the pH was adjusted to 2, and the mixture was extracted with ethyl acetate. The ethyl acetate extracts were discarded. The pH was adjusted to 7, and the resulting mixture was extracted four times with methylene chloride. The extracts were dried and concentrated to produce fraction 2, which contained NNAL released from NNAL glucuronide.

Fractions 1 and 2 were further purified by reverse-phase and normal-phase high-performance liquid chromatography, as described previously, for analysis by gas chromatography, mass spectrometry, and selected ion monitoring.[10] A slight modification was the addition of a retention-time marker, 3-pyridyl-1-propanol (Aldrich Chemical, Milwaukee), to the samples before normal-phase analysis. It eluted at 22 minutes. The fractions containing NNAL (retention time, 37 minutes) and iso-NNAL (retention time, 36 minutes) were collected and analyzed by gas chromatography and thermal-energy analysis.[10] Cotinine in urine was measured by radioimmunoassay.[17]

Representative chromatograms of urine samples from the men before and after exposure to sidestream smoke are shown in Figure 1. Evidence supporting the identity of NNAL, both free and released by β-glucuronidase hydrolysis of NNAL glucuronide, is based on its retention times on reverse-phase and normal-phase high-performance liquid chromatography, the retention time of NNAL trimethylsilyl ether on gas chromatography, and the detection of NNAL trimethylsilyl ether by gas chromatography with a thermal-energy analyzer as a nitrosamine-selective detector. In our previous study of NNAL and NNAL glucuronide in the urine of smokers, we confirmed the identity of NNAL trimethylsilyl ether by gas chromatography, mass spectrometry, and selected ion monitoring. In this study, the small quantities of NNAL and its glucuronide that were present in urine precluded other analyses. The mean proportion recovered was 72 percent for NNAL and 48 percent for NNAL glucuronide. The results in Table 1 have been corrected for recovery.

Each urine sample was analyzed once for NNAL, NNAL glucuronide, and cotinine. Results of the analyses of urine samples from the men before and after exposure are expressed as means (±SD). Statistical comparisons were carried out with t-tests (two-tailed).

Results

The results of the measurements of NNAL and its glucuronide in the 24-hour urine samples are shown in Table 1. In all the men for whom data were available, excretion of the metabolites increased after exposure to sidestream smoke.

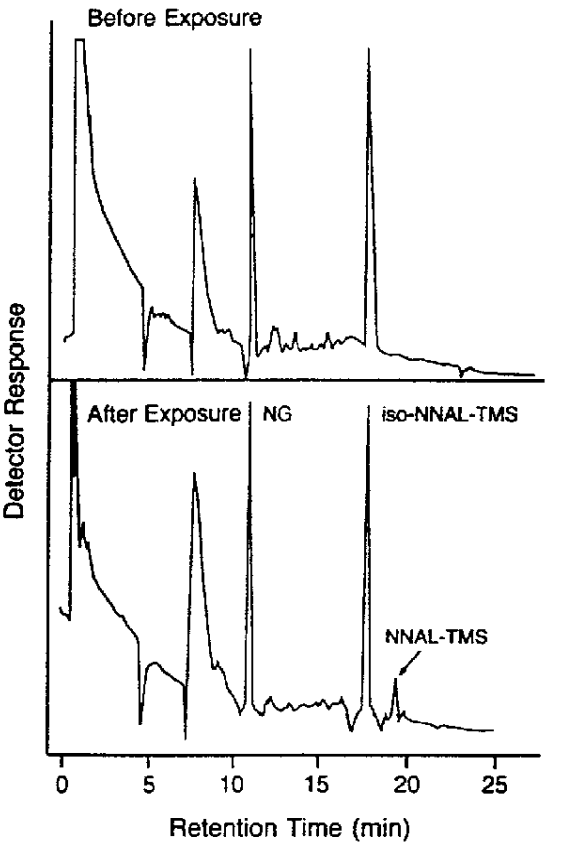

Figure 1. Chromatograms of Fraction 2 Containing NNAL Released from NNAL Glucuronide.

The results of gas chromatography and thermal-energy analysis are from Subject 1 in study 2. The internal standards nitrosoguvacoline (NG), added before injection, and iso-NNAL, added at the beginning of the analysis and detected as its trimethylsilyl derivative (TMS), are indicated.

In the two studies, seven measurements of urinary NNAL and its glucuronide were obtained after exposure, for six of which corresponding base-line results were also available. The mean amount of NNAL and its glucuronide was significantly higher in the seven measurements made after exposure than in the six made at base line (33.9±20.0 vs. 8.4±11.2 ng per 24 hours [127±74 vs. 31±41 pmol per day], $P<0.001$). The mean urinary excretion of cotinine was also significantly higher after exposure than before (89.6±35.9 vs. 8.9±9.7 μg per 24 hours [509±204 vs. 50±55 nmol per day], $P<0.001$). In the six cases in which NNAL and its glucuronide were measured both before and after exposure, the excretion of these metabolites after exposure to sidestream smoke increased by a factor of 10.2±12.8 (range, 2.2 to 35.4). The

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Table 1. NNAL and Its Glucuronide in the Urine of Men before and after Exposure to Sidestream Cigarette Smoke.*

Study and Subject No	NNAL†		NNAL Glucuronide‡		Cotinine§	
	Before	After	Before	After	Before	After
	ng/24 hr				μg/24 hr	
Study 1						
1	<0.6	3.6	—	—	<2	62
2	<0.6	6.0	—	—	<2	66
3	<0.6	15	<1.0	44.2	3.7	123
4	2.7	6.7	25.8	55.3	18	146
5	1.4	4.1	—	—	<2	18
Study 2						
1	<0.6	2.7	<1.0	15.1	<2	92
2	1.3	4.4	5.3	13.1	2.8	57
3	<0.6	4.0	1.4	12.3	4.8	65
4	2.1	5.6	11.8	35.0	24	96
6	—	3.7	—	20.3	4.8	48

*Six nonsmokers were exposed to sidestream smoke from Kentucky 2R1 reference cigarettes in a 16-m^3 room. Each study consisted of two 90-minute sessions. In session 1, 6 cigarettes were smoked, and in session 2, 12 cigarettes were smoked. Nicotine concentrations in the room (in micrograms per cubic meter) were as follows: study 1, session 1, 62; study 1, session 2, 230; study 2, session 1, 38; and study 2, session 2, 110. The NNK concentrations in the room (in nanograms per cubic meter) were as follows: study 1, session 1, 75, and study 1, session 2, 263. Dashes indicate that the values were not determined. In study 1, three samples of the NNAL glucuronide fraction were lost owing to technical problems. In study 2, Subject 6 had an interfering peak that prevented base-line quantitation of NNAL and NNAL glucuronide.

†To convert values to picomoles per day, multiply by 4.78. The limit of detection was approximately 0.6 ng of NNAL per 24-hour urine sample.

‡To convert values to picomoles per day, multiply by 2.59. The limit of detection was approximately 1 ng of NNAL glucuronide per 24-hour urine sample.

§Values include some 3′-hydroxycotinine. To convert values to nanomoles per day, multiply by 5.68. The limit of detection was approximately 2 ng of cotinine per milliliter of urine.

corresponding increase in urinary cotinine excretion in these six cases was 18.7±12.4 times (range, 4.0 to 33.2). The urinary excretion of NNAL and NNAL glucuronide and that of cotinine were correlated ($r = 0.89$, $P<0.001$) (Fig. 2).

In three cases — with Subject 4 in studies 1 and 2 and Subject 2 in study 2 — the base-line results indicated exposure to NNK. In Subject 4, the urinary cotinine values before both studies were also consistent with exposure to tobacco smoke. In all the other cases the base-line urinary excretion of NNAL or NNAL glucuronide was either below or close to the limit of detection.

In our previous study[10] of NNAL and its glucuronide in the urine of smokers, the molar ratio of those substances to cotinine was 1:3900. In this study, the corresponding ratio was 1:4600±1800. The mean 24-hour urinary excretion of NNAL and its glucuronide in the urine of 11 smokers was 4.0±1.7 μg per 24 hours (11±4 nmol per day), which, compared with the data from the current study, indicates that NNK uptake was about 120 times greater in smokers than in nonsmokers exposed to sidestream smoke.

Discussion

In this study, we demonstrated the uptake and metabolism of NNK by nonsmokers exposed to sidestream cigarette smoke. The metabolites identified in urine were NNAL and its glucuronide. NNAL, like NNK, is a potent pulmonary carcinogen in rats and mice, inducing a high incidence of adenocarcinoma of the lung.[7,14] Our demonstration that nonsmokers take up NNK thus provides support for the conclusion of the EPA that environmental tobacco smoke causes lung cancer in humans. It is notable that NNK and NNAL induce primarily pulmonary adenocarcinomas, as occurs in nonsmokers exposed to environmental tobacco smoke.[2,6,7,14]

The major lung carcinogens in cigarette smoke appear to be NNK and polynuclear aromatic hydrocarbons.[18] In indoor air polluted with tobacco smoke, the total concentrations of polynuclear aromatic hydrocarbons are similar to those of NNK.[16,19] The uptake of carcinogenic polynuclear aromatic hydrocarbons by nonsmokers after exposure to environmental tobacco smoke has not been reported.

The ratio of NNAL and NNAL glucuronide to cotinine in urine was similar in passive smokers (1:4600) and active smokers (1:3900). The concentrations of nicotine and NNK in the sidestream smoke of a Kentucky 2R1 cigarette are about twice those in mainstream smoke. A constant ratio of NNAL and NNAL glucuronide to cotinine could therefore be expected in the urine of passive and active smokers, an expectation consistent with our results. A previous study of 4-aminobiphenyl hemoglobin adducts in nonsmokers exposed to environmental tobacco smoke also demonstrated that biomarker levels in passive smokers were related to concentrations of 4-aminobiphenyl and nicotine in sidestream smoke.[4]

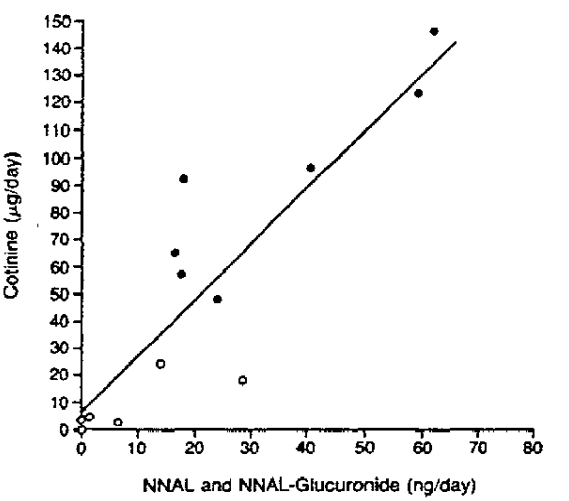

Figure 2. Relation between 24-Hour Urinary Excretion of NNAL and NNAL Glucuronide and Cotinine Excretion in Nonsmokers before (○) and after (●) Exposure to Sidestream Cigarette Smoke.

To convert values for NNAL to picomoles per day, multiply by 4.78; to convert values for NNAL glucuronide to picomoles per day, multiply by 2.59; and to convert values for cotinine to nanomoles per day, multiply by 5.68.

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The exposure to sidestream smoke in this study was comparable to that which might be encountered in a heavily smoke-polluted bar, given the nicotine concentrations in our exposure room and those reported previously in bars (up to 119 μg per cubic meter).[20,21] Most indoor environments would have lower concentrations of nicotine and NNK than those in our exposure room,[16,19,22,23] and the concentrations of NNAL and its glucuronide in the urine of people in those environments would probably be lower than the concentrations reported here. Our results nevertheless establish the principle that NNK is taken up and metabolized by nonsmokers who are exposed to sidestream cigarette smoke, and they therefore provide evidence supporting the link between exposure to environmental tobacco smoke and the risk of lung cancer.

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